

Exploring Biomedical Potential of Phytochemical Synthesized Iron Nanoparticles: *In Vivo* and *In Vitro* Evaluation

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Abstract

Nanobiotechnology has garnered immense attention across multidisciplinary fields, particularly for its biopharmaceutical applications. This study aims to synthesize iron nanoparticles (FeNPs) using an aqueous extract of *Murraya koenigii* (AE-Mk) and evaluate their biological activity. The initial synthesis was confirmed via ultraviolet–visible spectroscopy, which displayed an absorption band with a maximum wavelength at 620 nm. The contribution of AE-Mk to FeNPs synthesis was assessed using Fourier transform infrared spectroscopy, which identified various functional groups, including alcohol, N–H stretch, aliphatic primary amine, carboxylic acid, alkyne, and alkane/alkene. Characterization confirmed the elemental nature, amorphous form, and aggregate morphology of FeNPs, as observed through energy-dispersive X-ray, X-ray diffraction, and scanning electron microscopy, respectively. FeNPs exhibited a total phenolic content of 84.23 µg GAE/mg and a total flavonoid content of 78.62 µg QE/mg at 1 000 µg/mL. Additionally, they showed significant reducing ability (69.43%) and prominent scavenging potential against 2,2-diphenyl-1-picrylhydrazyl (80.63%), nitric oxide (73.01%), and hydrogen peroxide (71.62%) at 600 µg/mL. Strong antimicrobial activity was observed at 500 µg/mL against *Bacillus subtilis* (19 mm), *Escherichia coli* (16 mm), *Schizophyllum commune* (19 mm), and *Aspergillus niger* (13 mm). The biological compatibility of FeNPs was evaluated in Wistar rats, showing no adverse effects at the administered dose (50 µg/mL). Furthermore, *in vitro* anticancer activity at 100 µL showed significant cell viability loss (58.23%, IC₅₀: 51.35) against HeLa cell lines. These findings highlight the dynamic potential of FeNPs for biomedical applications.

Keywords: nanobiotechnology; synthesis; characterization; cell viability; inhibition; biomedical

Introduction

Biomedical applications require formulations with specific active substances designed to achieve precise

size and shape [1–3]. Nanotechnology facilitates the synthesis of nanomaterials that are easy and efficient to produce. In this context, inorganic metal nanoparticles (particularly those <100 nm) provide a

valuable resource for scientists exploring applications in the medical field [4]. Interest in metal nanoparticles has increased in recent years [5] due to their unique physicochemical properties, including optical, electrical, chemical, and photochemical characteristics [6]. Iron nanoparticles (FeNPs) have gained significant attention for their diverse biological applications [7-9], such as antimicrobial, antidiabetic, and anticancer therapies, as well as bioimaging, bio-labeling, drug delivery, water remediation, photoablation, and hyperthermia treatments [10,11].

Various methods are used to synthesize metal nanoparticles, including physical, chemical, and biological approaches [12]. Although physical and chemical methods can synthesize nanoparticles with accurate size and shape, they require expensive and toxic chemicals as well as high temperatures and pressure, which makes them unsuitable for biological applications [13]. Conversely, biological synthesis methods utilizing bacteria, fungi, algae, animals, or plants have garnered significant interest [14]. While microorganisms are challenging to culture and process [15], plant extracts have gained popularity due to their nontoxic, rapid, inexpensive, and straightforward approach [16]. Notably, plant extracts contain secondary metabolites with bioactive compounds that facilitate nanoparticle synthesis by acting as reducing, capping, and stabilizing agents [17]. Optimizing environmental factors such as plant extract volume, salt concentration, reaction pH, temperature, and time can enhance the quality and stability of plant-based metal nanoparticles [18].

Cancer is an uncontrolled growth of cells that arises from various factors, including genetic variations and external stimuli [19]. It remains a life-threatening disease with high global mortality. Despite significant advancements, including genetic-level interventions, complete eradication remains challenging, particularly in its fourth stage. Recently, nanobiotechnology, integrating nanotechnology, biotechnology, and biological tools, has played a crucial role in assisting researchers to introduce novel therapeutic approaches [20]. These approaches can potentially uncover critical insights for more effective treatment [3, 21]. Owing to their remarkable properties, metal nanoparticles have emerged as promising candidates in nanobiotechnology for cancer therapy.

Despite the availability of a broad range of antimicrobial drugs, the medical field continues to search for novel agents owing to drug-resistant pathogens [22]. Besides, many of these drugs are associated with adverse side effects; therefore, demand alternative. Current biomedical research is focused on developing formulations with minimal toxicity and maximum biocompatibility [23]. Numerous studies have reported the antimicrobial properties of green-synthesized metal nanoparticles [24]. Metal nanoparticles exhibit antimicrobial activity through various mechanisms, and their potency varies depending on the plant extract used in their synthesis [25]. Similar to antimicrobial, these nanoparticles exhibit strong free radical scavenging ability, with increasing concentrations enhancing their effectiveness against reactive oxygen species (ROS) and free radicals, thereby demonstrating significant antioxidant potential as well [26].

Murraya koenigii, a medicinal plant from the Rutaceae family, is native to South Asia but is also cultivated in many other regions [27]. It is well known for its distinctive aroma and diverse properties [28]. In some cultures, it is considered the “spirit of food” due to its ability to enhance flavor [29]. Although the entire plant possesses medicinal value, the leaves exhibit the most significant curative properties [30], including antioxidant, antidiabetic, anticancer, antifungal, antibacterial, anti-inflammatory, antidepressant, and anthelmintic activities [31]. Key compounds such as mahanimbine and koenigine, present in the leaves, contribute to the plant’s anticancer potential [32]. Moreover, the leaves aid in blood purification, alleviate kidney pain, and are used to treat eruptions and bruises [33].

To the best of our knowledge, this is the first report on FeNPs synthesis using the medicinal plant *M. koenigii* through an ecofriendly approach. The synthesis process was optimized based on key environmental variables. The nanoparticles were characterized using various analytical techniques, including ultraviolet–visible (UV–Vis) spectroscopy, Fourier transform infrared (FTIR) spectroscopy, scanning electron microscopy (SEM), energy-dispersive X-ray (EDX), and X-ray diffractometer (XRD), to assess their structural integrity. To evaluate their biomedical potential, antimicrobial, phenolic content, antioxidant, hemolytic, and cytotoxicity (*in vitro*), as well as biological compatibility (*in vivo*) studies, were conducted.

Experimental

Chemicals

Iron(III) chloride hexahydrate ($\text{FeCl}_3 \cdot 3\text{H}_2\text{O}$) (99.9%), quercetin, Folin–Ciocalteu reagent (FCR), nitric oxide (NO), methanol, sodium nitroprusside, sodium carbonate, sodium hydroxide, ascorbic acid (vitamin C), gallic acid (GA), 2,2-diphenyl-1-picrylhydrazyl (DPPH), aluminum trichloride, trichloroacetic acid, potato dextrose agar (PDA), Mueller–Hinton agar (MHA), Mueller–Hinton broth (MHB), butylated hydroxytoluene (BHT), hydrogen peroxide (H_2O_2), phosphate buffer, potassium ferricyanide, chloramphenicol, fluconazole, deionized water, sodium phosphate, sulfuric acid, ammonium molybdate, heparin, phosphate-buffered saline (PBS), Triton X-100, paraffin, formalin, hematoxylin and eosin, dimethyl sulfoxide (DMSO), MTT salt [(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide], and Dulbecco's Modified Eagle Medium (DMEM) were all of analytical grade, procured from Merck (Sigma-Aldrich), and used without further purification.

Synthesis protocol

To prepare AE-Mk, fresh *M. koenigii* leaves were thoroughly washed with tap water, followed by a final washing with double-distilled water, and then air-dried. The dried leaves were cut into medium-sized pieces and heated (6 g/100 mL) over a burner flame for 15 min. The resulting solution was cooled and filtered through 0.25- μm filter paper. FeNPs were synthesized using a freshly prepared 0.1 M iron(III) or FeCl_3 solution in deionized water, followed by 25 min of sonication at room temperature to ensure homogeneity. The solution was stirred for 15 min using a magnetic stirrer before AE-Mk (6 mL) was added dropwise. Initially, the solution's color changed from rust to reddish-brown during the gradual addition of the yellow extract. After 15–20 min of stirring, the solution was kept in the dark and monitored for synthesis completion using UV–Vis spectroscopy, following a modified method from Ningthoujam [34].

Optimization study

The synthesis process was optimized by varying the extract volume (10–100 mL), salt concentration (0.001–0.5 mol/L), pH (3–9), temperature (25–60 °C), and incubation time (5 min to 24 h), following the method described in Ref. [35].

Characterization techniques

The synthesis of colloidal FeNPs was confirmed using UV–Vis spectroscopy (UV-1800, Shimadzu spectrophotometer) within the 300–800 nm wavelength range, with a resolution of 1 nm [36]. For FTIR, XRD, EDX, and SEM analyses, the FeNPs were used in powdered form. The FeNP solution was purified via centrifugation at 4 000 r/min for 15 min, after which the supernatant was discarded. The residue was resuspended in deionized water, and the process was repeated multiple times. Finally, the mass was oven-dried at 60 °C and ground into a powder. FTIR spectroscopy (4 000–500 cm^{-1} , transmittance mode) was used to identify functional groups in AE-Mk and FeNPs. A dried pellet of KBr was used, and a resolution of 4 cm^{-1} was selected [37]. XRD (X'Pert X' Celerator diffractometer) was employed to analyze the texture, using a 2θ configuration with a nickel monochromator, covering the 20°–80° range and $\text{Cu-}k_\alpha$ radiation ($\lambda = 1.54 \text{ \AA}$). The sample was placed on a copper-coated grid for analysis. The mean crystal size was calculated using Scherrer's equation ($D = 0.9 \lambda / \beta \cos \theta$) [38]. The elemental composition of the FeNPs was analyzed using EDX (Sigma), as described by Yassin [39]. Finally, SEM (Hitachi S-2500C) was used to assess the surface characteristics of the FeNPs. A thin film of the FeNP colloidal suspension was prepared by spreading it on a glass rod and oven-drying it [40].

Biological assays

Total phenolic content (TPC)

The TPC in FeNPs and AE-Mk at concentrations of 25, 50, 100, 250, 500, and 1,000 $\mu\text{g/mL}$ was determined using the method proposed by Popoola [41]. Sample solutions (5 mg/mL) were sonicated for 10–20 min at room temperature before use. The test tubes containing these sample solutions were treated with 2 mL of 10% FCR and incubated in the dark for 5 min. After adding 2 mL of 5% Na_2CO_3 solution, the tubes were incubated in the dark for 2 h. Absorbance was measured at 765 nm using an ELISA plate reader and a UV–Vis spectrophotometer. TPC was estimated using a standard curve generated with GA, and the results were expressed as $\mu\text{g GAE/mg}$ (GAE, gallic acid equivalents).

Total flavonoid content (TFC)

The colorimetric method was employed to determine the TFC in FeNPs and AE-Mk. Samples (50 μL , sonicated) from various concentrations (25, 50, 100,

250, 500, and 1 000 µg/mL) were placed in separate test tubes, followed by the addition of equal volumes of deionized water and 50 µL of 5% sodium nitrate, with shaking. The samples were incubated and then reacted with 50 µL of 5% aluminum chloride solution and kept in the dark for 10 min. After incubation, the mixtures were mixed with deionized water and sodium hydroxide solution. The absorbance of the solutions was measured at 510 nm. A calibration curve was constructed using a quercetin standard, and the results were expressed as µg QE/mg (QE, quercetin equivalent).

Reductive potential activity (RPA)

Sample solutions with concentrations of 100, 200, 300, 400, 500, and 600 µg/mL were prepared in separate test tubes and treated with 2.5 mL of phosphate buffer (pH 6.6). The mixture was then reacted with 2.5 mL of 1% potassium ferricyanide and incubated at 50 °C for 15 min. After incubation, 2.5 mL of 10% trichloroacetic acid was added, and the mixture was incubated for an additional 15 min. The resulting supernatant (2.5 mL) was treated with 0.5 mL of ferric chloride, and the absorbance was measured at 700 nm using a spectrophotometer. Ascorbic acid was used as the positive control, and deionized water was used as the negative control [42].

DPPH assay

The antioxidant activity of FeNPs, AE-Mk, and ascorbic acid at concentrations of 100, 200, 300, 400, 500, and 600 µg/mL was assessed using the DPPH assay, following a modified method by Chandrasekar [43]. A freshly prepared methanolic DPPH solution (0.1 mmol/L) was added to 1 mL of FeNPs and the extract, which were treated under the same conditions as the FeNPs. The resulting solution was incubated for 30 min at room temperature. Ascorbic acid was used as the standard for comparison. Absorbance was measured at 517 nm using deionized water as the blank. The DPPH scavenging ability of the samples was calculated using Eq. (1).

$$\text{Inhibition (\%)} = 100 - \frac{\text{absorbance of control} - \text{absorbance of sample}}{\text{absorbance of control}} \times 100 \quad (1)$$

H₂O₂ assay

The H₂O₂ scavenging activity of FeNPs and AE-Mk

was evaluated using a modified version of Patel's method [44]. Sample solutions were prepared at different concentrations (100, 200, 300, 400, 500, and 600 µg/mL) and mixed with 2.4 mL of 0.1 mol/L phosphate buffer (pH 7.4) and 0.6 mL of 40 mmol/L H₂O₂. The mixture was vortexed and incubated at room temperature for 10 min. Absorbance was measured at 610 nm using deionized water as the blank. Ascorbic acid served as the positive control. Scavenging potential was calculated using Eq. (1).

NO assay

Equal volumes of test solutions at different concentrations were mixed with 20 mM sodium nitroprusside and incubated at room temperature for 1 h. Absorbance was measured at 546 nm, using BHT as a positive control and deionized water as the negative control. Inhibition (%) was calculated using Eq. (1), following the method described by [45].

Hemolytic activity

The effect of FeNPs on the red blood cells (RBCs) and hemoglobin release was assessed using a method adapted from a study [46]. Blood was collected from a healthy human donor, treated with heparin to prevent coagulation, and centrifuged at 1000 r/min for 10–15 min. The supernatant was carefully removed, leaving a pellet that was resuspended in chilled PBS (pH 7.4). FeNP solutions at different concentrations (10, 20, 30, 50, 75, and 100 µg/mL) were prepared in separate test tubes, each containing 5% (v/v)/FeNPs, and adjusted to a final volume of 1 mL. The mixtures were incubated in a shaker at 37 °C for 1 h under the same conditions. Absorbance of the resulting supernatant was measured at 540 nm. Triton X-100 served as the positive control, while PBS was used as the negative control. Hemolytic activity was calculated using Eq. (2).

$$\text{Hemolysis (\%)} = A = \pi r^2 \times \frac{\text{absorbance of sample} - \text{absorbance of PBS}}{\text{absorbance of Triton X-100} - \text{absorbance of PBS}} \times 100 \quad (2)$$

Antimicrobial assay

The agar well diffusion method was employed to determine the antimicrobial activity of FeNPs and AE-Mk against *E. coli*, *B. subtilis*, *A. niger*, and *S. commune* microbial strains. Microbial strain cultures were obtained from the Department of Biochemistry, UAF, and subcultures were prepared and maintained

at 4 °C. Sterilized Petri plates were autoclaved, oven-dried for 15 min, and filled with freshly prepared MHB and PDA. A 200 µL aliquot of pure microbial cultures (10^6 CFU/mL) was evenly spread onto the plates using a sterile cotton swab. After 10–15 min, wells approximately 6 mm in diameter were created in the gel medium using a sterile well borer. The synthesized FeNPs and AE-Mk at different concentrations of 50, 100, 200, 300, 400, and 500 µg/mL (50 µL per well) were introduced into these wells. Chloramphenicol (standard) served as the positive control for antibacterial activity, fluconazole for antifungal activity, and deionized water as the negative control. The plates were incubated at 37 °C for 24 h for bacterial cultures and at 28 °C for 48 h for fungal cultures, after which the inhibition zones were measured [39].

***In vivo* biological compatibility study**

The effects of FeNPs at doses of 30, 50, and 100 µg/mL were evaluated in Wistar rats (4–6 months old, approximately 150 g), which were thoroughly examined for injury and illness before the study. The animal procedure was approved and screened based on the ethical guidelines of the National Biosafety Committee and the Punjab Biosafety Rules, 2014, with approval from the Office of Research, Innovation & Commercialization, University of Agriculture, Faisalabad (Bioethics Certificate No. Chem 1321). The animals were obtained from a designated facility for growth and protection and housed in the “Animal Warehouse” at the University of Agriculture under standard conditions of diet, lighting, and others, following International standard operating procedures (SOPs) provided by the International Laws for the welfare and protection of animals and their whereabouts.

Three animals were assigned to each group: FeNPs-treated groups and the control group (PBS). They were closely monitored under established SOPs. The experimental period spanned 28 days, with doses administered every 2 days through tail vein injection, totaling 13 doses. On day 28, the animals were sacrificed for liver enzyme analysis (through blood collection) and histopathological examination of major organs.

Liver enzyme functionality test

Liver enzyme integrity was evaluated by measuring alkaline phosphatase (ALP), serum glutamic pyruvate transaminase (SGPT), aspartate transaminase (AST),

and serum bilirubin (total, direct, and indirect) using the standard kit protocol. Blood samples were centrifuged at 1 000 rpm for 15 min before analysis.

Sample preparation for histopathology

Organ samples were collected through dissection and preserved in freshly prepared 10% formalin. The fixed tissues were embedded in paraffin, stained with hematoxylin and eosin, and examined under an optical microscope at 40× magnification, with images captured for analysis.

***In vitro* cytotoxicity**

MTT assay

In vitro cytotoxicity testing was performed at the HEJ Institute, an International Center for Chemical and Biological Sciences. The MTT assay was performed following Venkatesan [47], with modifications. HeLa cell lines were procured, cultured in DMEM under standard conditions, and assessed for cytotoxicity using MTT salt. Cell maintenance and culture were performed in DMEM supplemented with 10% fetal calf serum, 75 mg/mL streptomycin, 120 U/mL penicillin, and amphotericin. Humidity was regulated with CO₂ at 37 °C. FeNPs sample solutions and stock solutions were prepared in DMSO at the desired concentrations. Cell pellets were collected through centrifugations of FeNPs and trypsin at 3 000 r/min. Increasing concentrations (30–100 µg/mL) of the sample solution in a 200 µL volume were added to a 96-well microplate and incubated under 5%CO₂ at 37 °C for 24 h in DMEM. Subsequently, the samples were aspirated, and the MTT assay was performed. The aspirated samples were treated with 10% MTT (200 µL) in the microplate, with the final concentration adjusted to 0.5 mg/mL, followed by a further 3-h incubation under the same conditions of temperature and pressure. The supernatant was then removed, and the resulting formazan crystals were treated with 100 µL of DMSO solution. Untreated cells served as controls. The absorbance of test samples was measured at 630 nm, and cytotoxicity was calculated based on the absorbance results.

Inhibition (%) =

$$100 - \frac{\text{mean absorbance of test sample}}{\text{mean absorbance of positive control}} \times 100 \quad (3)$$

Statistical analysis

All experimental data were analyzed from at least five replicates and expressed as the mean ± S.E.

Differences among variables were assessed using one-way ANOVA (GraphPad Prism 5.0), with significance set at $p < 0.05$ and $p < 0.001$.

Results and Discussion

Synthesis

Absorption spectroscopy is used to analyze the optical behavior of green-synthesized metal nanoparticles, as the absorption profile serves as a preliminary indicator of the synthesis process. The absorption spectra of FeNPs, along with AE-Mk and iron salt, are presented in Fig. 1. The spectra showed an absorption band at 620 nm, attributed to surface plasmon resonance (SPR). SPR arises from the interaction between metal nanoparticles and light, imparting unique optical properties to the nanoparticles. This phenomenon occurs when the frequency of surface electrons in the metal matches that of the incident photons of the beam when exposed to light energy, leading to electron excitation and oscillation. The size, shape, and morphology of biosynthesized metal nanoparticles influence the red or blue shift in wavelength, as indicated by the optical spectra. Additionally, the dielectric medium also influences the synthesis process. The colloidal solution was monitored at regular intervals until synthesis was complete, which occurred within 4 h. Previous studies, including those by Biswal and Misra [48], have reported and explained the synthesis process of metal nanoparticles based on the SPR phenomenon.

Optimization of factors

The optimization study was conducted to investigate the controlled size, shape, and morphology of FeNPs for precise applications. Key environmental

variables—iron salt solution concentration, AE-Mk volume, temperature, pH, and time—were optimized during this study.

The trial on extract volume indicated that increasing the AE-Mk volume accelerated and enhanced synthesis, but only up to a specific limit (Fig. 2(a)). The band shifting from 610 to 630 nm indicated increased nucleation, resulting in larger FeNPs. Therefore, the optimal volume was selected as 75 mL, corresponding to 610 nm, where the best synthesis was observed. During analysis, it was noted that increasing the extract volume accelerated the synthesis process by providing more biomolecules for reduction. As more biomolecules became available in the medium, the synthesis rate accelerated.

The size and shape of the synthesized nanoparticles were significantly influenced by the extract volume. Similarly, the synthesis rate, size, and shape of metal nanoparticles were influenced by the nature and type of biomolecules present in the plant extract, as different extracts contribute differently to the reduction process, as previously reported by Maniam [49] and Akintelu [50]. The iron salt concentration ranged from 0.001 to 0.5 mol/L, significantly affecting FeNP size distribution, with no obvious synthesis at lower concentrations.

Conversely, broader bands were observed at high salt concentrations (Fig. 2(b)). While the precursor enhanced the synthesis rate, high concentrations led to agglomeration. Iron salt concentration favored the formation of smaller nanoparticles at a slower rate; however, the size was increased at high salt concentration due to nanoparticle accumulation and an accelerated synthesis rate [51]. Therefore, 0.1 M was selected as the optimal concentration for effective synthesis.

The effect of temperature was crucial, as higher reaction temperatures significantly reduced synthesis time. A noticeable change in solution color occurred with increasing temperature, indicating its role in facilitating interaction between the precursor and reducing agent. Temperature variation from 25 to 60 °C was examined, showing that beyond a specific limit (Fig. 2(c)), aggregation led to particle size enlargement. Consequently, 25 °C (620 nm) was selected as the optimal temperature. Synthesis stability and velocity were directly influenced by temperature, affecting not only nanoparticle shape and size but also accelerating the reaction rate [52].

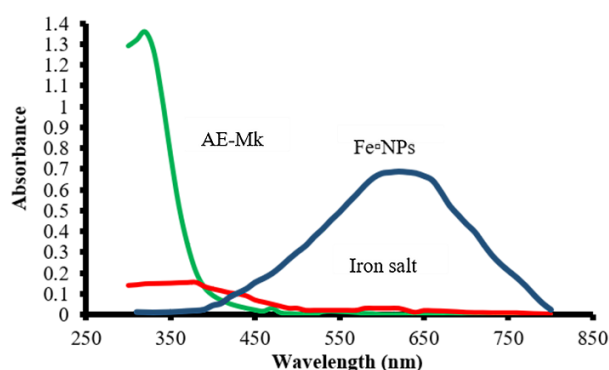


Fig. 1 UV-Vis absorbance profile of FeNPs, AE-Mk, and iron salt as a function of wavelength.

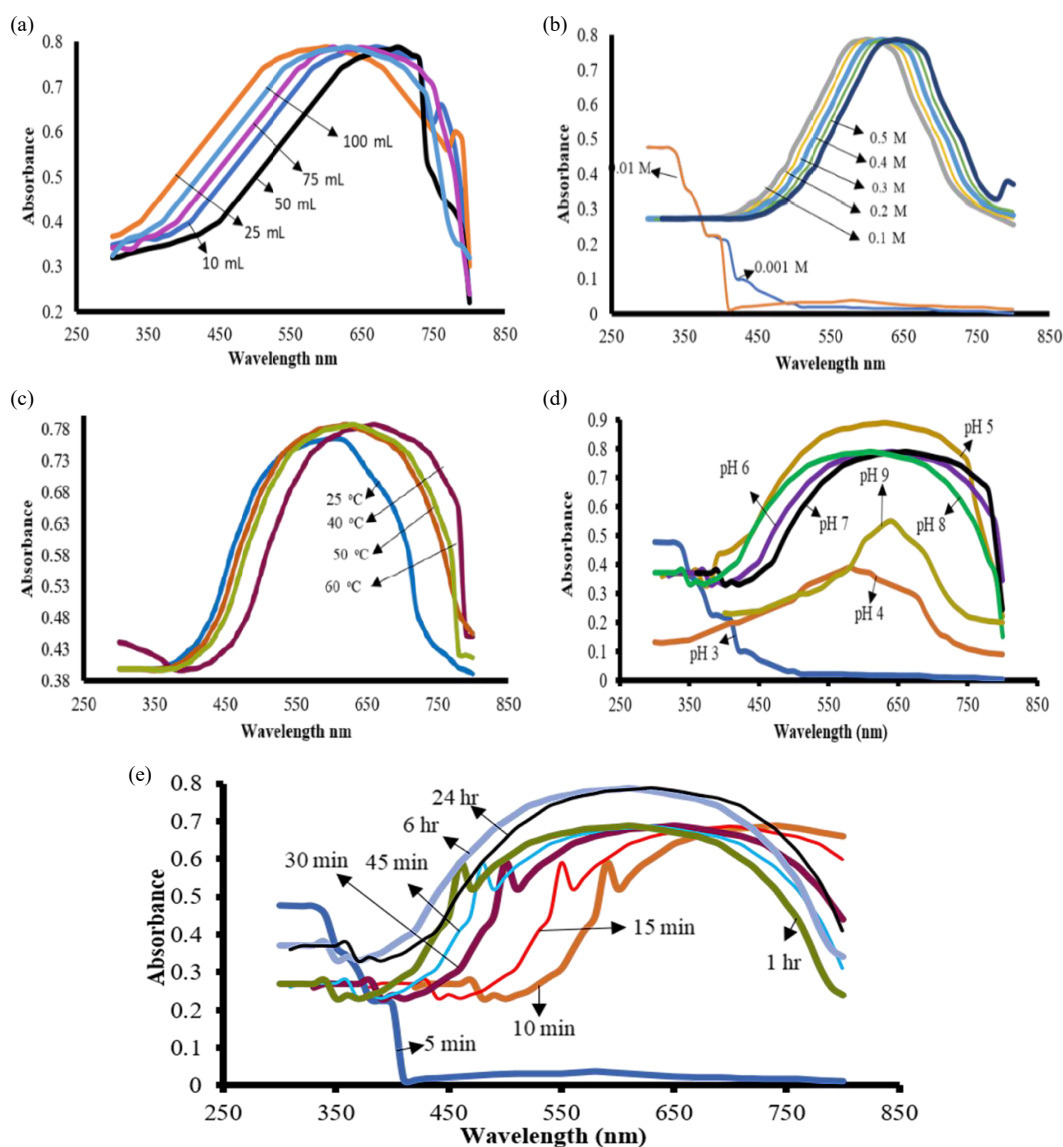


Fig. 2 Effect of environmental variables on FeNP synthesis: **(a)** AE-Mk volume, **(b)** iron salt concentration, **(c)** temperature, **(d)** pH, and **(e)** reaction time. Each reaction mixture was repeated five times, with results presented as mean $\pm$ S.E.

However, excessively high temperatures led to supersaturation, compromising synthesis quality.

The effect of pH variation was crucial, as results showed significant changes in the synthesis process across a pH range of 3–9 under identical conditions (Fig. 2(d)). Reaction pH significantly influenced the synthesis rate, with an acidic reaction environment slowing the synthesis, as reported by Vincent [53], whereas neutral and slightly alkaline environments enhanced it. Optimal synthesis was observed at pH 8, where smooth band formation occurred at 610 nm. Additionally, reaction pH affected the synthesis rate and nanoparticle size, with larger FeNPs forming under acidic conditions and smaller ones produced in neutral or slightly alkaline medium within a short duration.

The time required for synthesis was monitored after mixing the two solutions and measured up to 24 h. The results showed that synthesis was complete within 6 h, with no significant changes in bandwidth or maximum wavelength observed thereafter (Fig. 2(e)). The structural morphology of the green-synthesized nanoparticles was strongly influenced by incubation time, consistent with findings reported by Ref. [54] on the effect of reaction time during FeNP synthesis.

Structural characterization

FTIR analysis

Understanding the role of biomolecules from AE-Mk in FeNP synthesis is essential for elucidating the reduction process. FTIR spectra revealed various

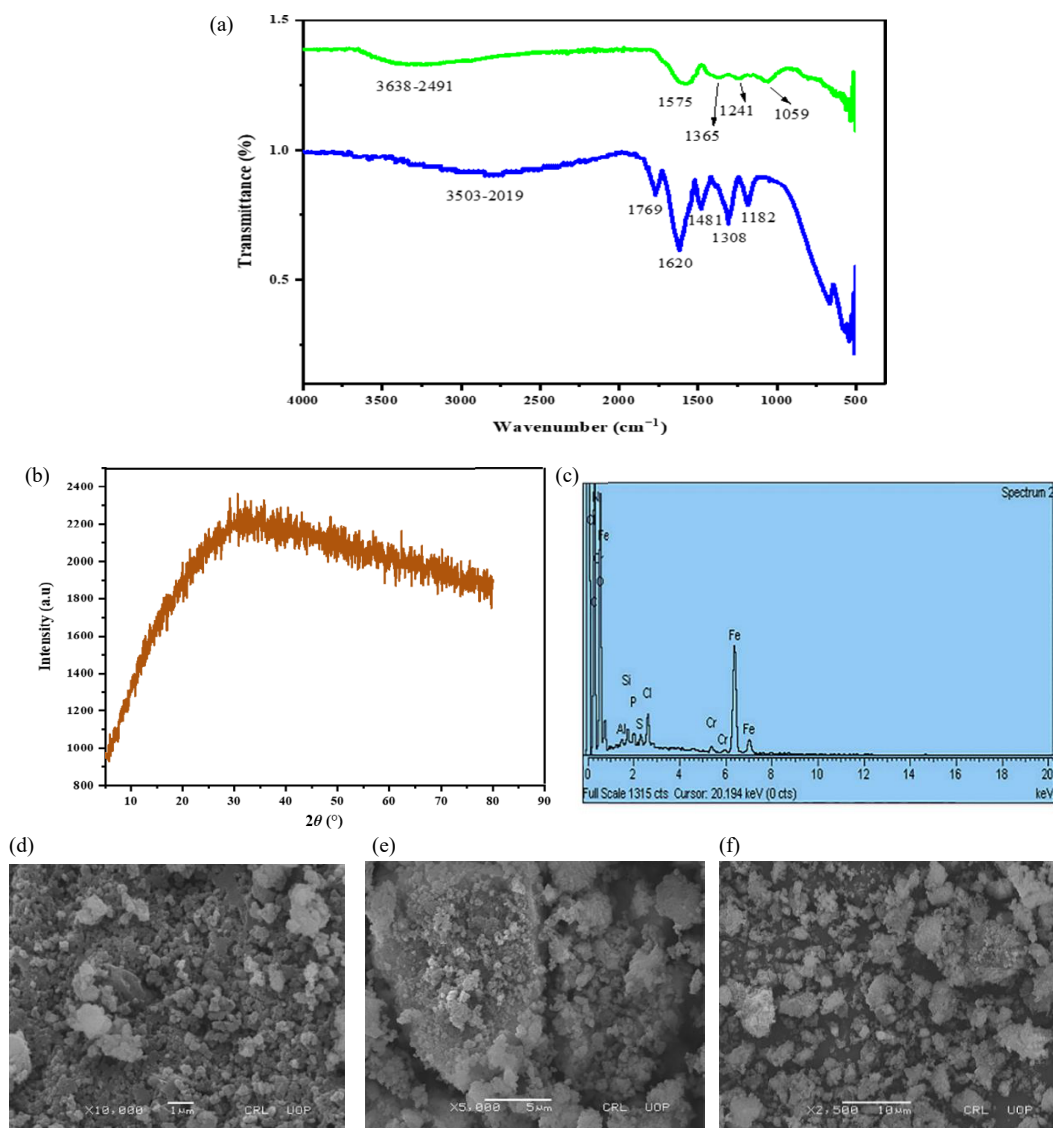


Fig. 3 FTIR spectra of (a) FeNPs (green line) and AE-Mk (blue line); (b) XRD image; (c) EDX analysis; and SEM micrographs of FeNPs at (d) 10 000 $\times$, (e) 5 000 $\times$, (f) 2 500 $\times$ magnification.

functional groups with different peak intensities (Fig. 3(a)). A comparative analysis between the FTIR spectra of FeNPs (green line in Fig. 3(a)) and AE-Mk (blue line in Fig. 3(b)) showed closely matching peaks, with slight shifts from the origin (from AE-Mk spectra) due to the bioreduction phenomenon responsible for FeNP synthesis. FTIR analysis provided clear evidence of the function of AE-Mk groups in the fabrication and stabilization of FeNPs (Fig. 3(a)). For instance, the AE-Mk showed a broad band at 3 503–2 019 cm^{-1} (phenol, N–H/C–H/O–H stretching vibrations of amines, amides, –COOH, and C–N groups). Prominent peaks were observed at 1 769 cm^{-1} (carbonyl functional groups), 1 620 cm^{-1} (C=C stretching), and 1 481, 1 308, and 1 182 cm^{-1} (associated with aliphatic ethers). In FeNPs, the band position shifted slightly from 3 638 to 2 491 cm^{-1} ,

indicating the rearrangement of functional groups such as alcohols, N–H stretches (primary amines), aliphatic primary amines, carboxylic acids, alkynes, alkanes, and alkenes.

These wavelength variations likely contributed to the reduction of iron ions (Fe^{3+} to Fe^0). Additional peaks at 1 575 and 1 241 cm^{-1} were attributed to weak N–H bending and C–N amine functional group, respectively. Similar observations were reported by Hasnain [55] during the synthesis of silver nanoparticles.

XRD analysis

An amorphous structure was observed in the XRD images of FeNPs, indicating extensive biomolecule coverage on the iron surface, forming a layer over it. However, a small diffraction peak of 44.9° (2θ)

represents the the presence of stabilized FeNPs. Due to the complete coverage of the outer surface by biomolecules, distinct peaks were not visible, except for a broad shoulder at 25° (Fig. 3(b)). Similar findings were reported by [56], who attributed the amorphous nature of synthesized FeNPs to the presence of abundant plant residues that mask the inner surface. These results support our analysis.

EDX analysis

The synthesized nanoparticles were analyzed using EDX to determine their structure (Fig. 3(c)). Several peaks were observed at different energy levels in the EDX image, indicating the presence of elements other than iron. Three distinct and intense peaks correspond to elemental iron at various energy levels, while others showed the relative abundance of chlorine, likely due to the biomolecules involved in the synthesis process. Furthermore, elements such as silicon and chromium were also observed in the sample. The EDX analysis was performed by Devatha and colleagues [57] on FeNPs synthesized from various extracts showed similar results, supporting our findings.

SEM analysis

SEM micrographs revealed that FeNPs had a polydispersed nature with rod-like, hexagonal, or slightly spherical shapes (Figs. 3(d)–3(f)). Aggregation was also observed, indicating the presence of plant extract, which covered the iron surface and played a crucial role in the synthesis process. Similar findings were reported by researchers such as Lin [58], who synthesized FeNPs using plant extracts and obtained results consistent with our findings.

Bioactivities

Phenolic determination

During different body functions, free radicals or ROS with one or more unpaired electrons are generated as byproducts and can develop strong oxidizing properties. While these species are necessary to perform various vital roles in immune function, chemical signaling, detoxification, and energy supply, they are also highly reactive and unstable. Due to their extreme reactivity, they can damage cells by scavenging electrons from biomolecules. Antioxidants help mitigate this damage by performing key physiological functions, such as breaking down peroxides, scavenging free radicals, suppressing chain

initiation and propagation, stimulating reducing potential, and inhibiting hydrogen abstraction through various pathways.

The TPC study performed on the FeNPs showed remarkable enhancement total phenolic content (Fig. 4(a)). With the increase in concentration from 25 to 1 000 µg/mL, TPC was observed from 22.98 to 84.23 µg GAE/mg. Under similar conditions, elevated values of TFC were observed from 13.63 to 78.62 µg QE/mg. In the case of AE-Mk, similar increase in (15.06–47.03 µg GAE/mg and 10.54–66.54 µg QE/mg) were observed for TPC and TFC, respectively. The higher TPC and TFC represented the role and involvement of the functional groups of AE-Mk and the significant development of the phenolic distribution in the green-synthesized FeNPs. Relevant reports presented by Mittal et al. [59] on the phytoconstituents of the plant extracts in terms of TPC and TFC demonstrated their potential presence in the synthesis of the nanoparticles.

RPA

FeNPs showed a prominent RPA, with values ranging from 14.06% to 69.43% as the concentration increased from 100 to 600 µg/mL (Fig. 4(b)). A similar values was found for AE-Mk, with RPA value ranging from 12.13% to 60.46% at higher concentrations. In comparison, vitamin C showed higher RPA values (20.65%–73.34%) under the same conditions. RPA is the potential of the substance to donate electrons to other species, particularly ROS or free radicals, converting them into nonradical forms. The color change during the reaction indicates the conversion of ferric to ferrous ions. The green synthesis pathway enhances the reducing properties of nanoparticles, making them effective in scavenging radicals. Sudha et al. [60] analyzed RPA in the sample of silver nanoparticles synthesized from the extract of *Lippia nodiflora*, and the observed values were quite comparable, supporting the validity of our findings.

DPPH assay

The DPPH potential of the FeNPs synthesized from the medicinal plant (*M. koenigii*) was significantly enhanced, with values ranging from 28.74% to 80.63% as the concentration increased. The inhibitory potential shown by AE-Mk was also appreciable, with values ranging from 22.32% to 70.34% as the concentration rose from 100 to 600 µg/mL. However, ascorbic acid showed more potential to scavenge free

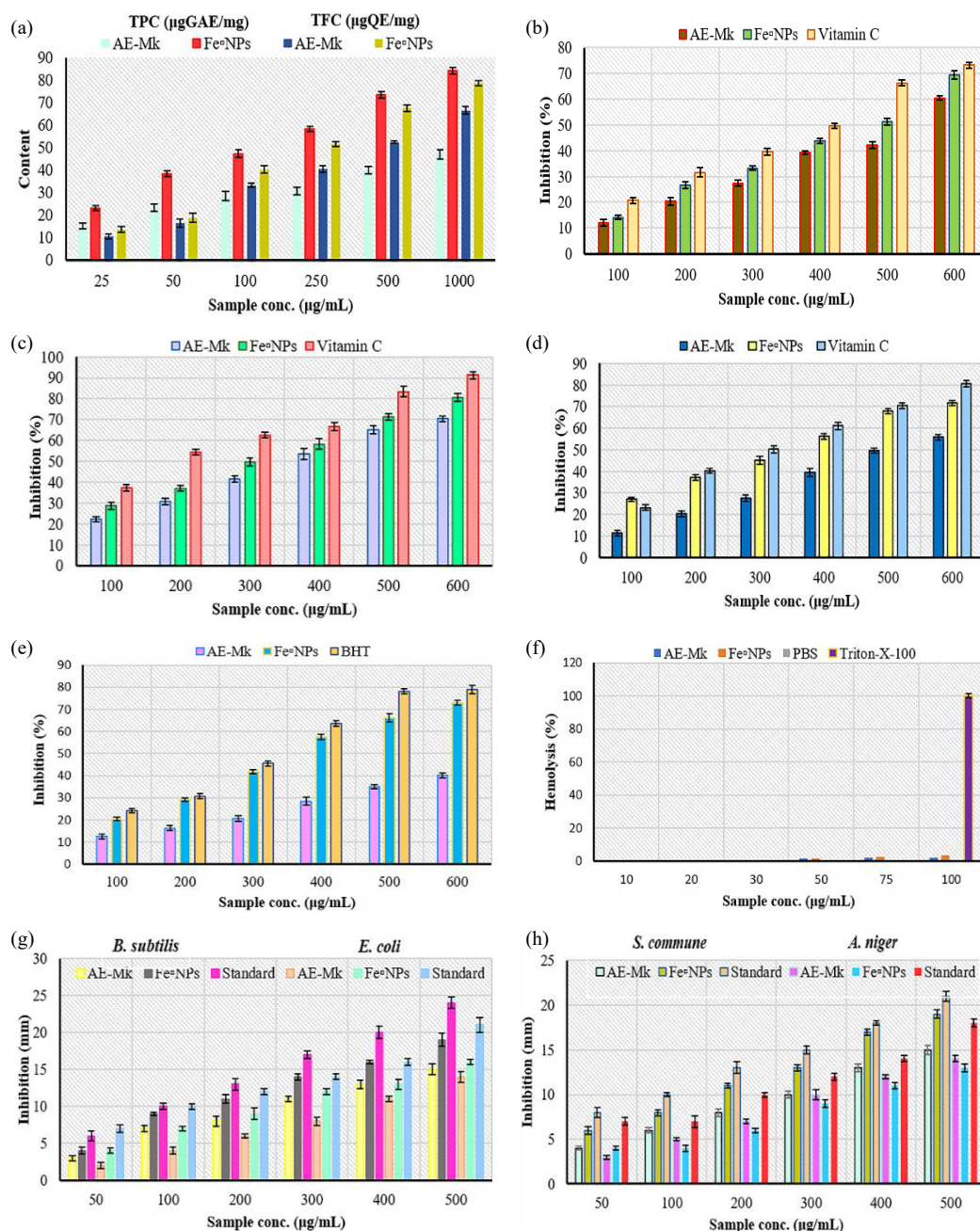


Fig. 4 Phenolic content (a) TPC and TFC; Antioxidant activity (b) RPA, (c) DPPH, (d) NO, and (e) H₂O₂ at different concentrations. (f) Hemolytic activity; (g) Antibacterial activity and (h) Antifungal activity against *E. coli*, *B. subtilis*, *S. commune*, and *A. niger* strains, compared to standards. Each value represents the mean $\pm$ S.E ($n = 5$), with significance levels of FeNPs compared to AE-Mk and the standard ($p < 0.05$; $p < 0.001$).

radicals, with DPPH values ranging from 37.46% to 91.21%, as shown in Fig. 4(c). The DPPH assay is a key marker of free radical scavenging potential. Yousaf et al. [61] reported significant DPPH scavenging activity in the methanolic extract of *A. millefolium*, mainly due to its phytoconstituents.

H₂O₂ assay

A significant scavenging potential of FeNPs was observed against H₂O₂ radicals, with values ranging from 27.09% to 71.62%. In comparison, AE-Mk had

a scavenging potential of 11.31%–55.82% at concentrations of 100–600 $\mu\text{g}/\text{mL}$, while vitamin C showed a range of 23.23%–80.64% (Fig. 4(d)). The experiments revealed that H₂O₂ can penetrate biological membranes and interact with the ferrous or cuprous ions, forming hydroxyl radicals. It can deactivate a specific enzyme through the oxidation thiol groups. Although H₂O₂ is a weak oxidizing agent, it can still inactivate some enzymatic functions, resulting in toxicity [62].

NO assay

The increased concentration of FeNPs enhanced the scavenging of NO radicals, with scavenging efficiencies ranging from 20.48% to 73.01% at 100–600 µg/mL concentrations, whereas AE-Mk exhibited inhibition rate between 12.54% and 40.08% at the same concentrations (Fig. 4(e)). In comparison, standard BHT exhibited a scavenging potential of 24.21%–79.02%. Mata et al. [63] reported a similar increase in the NO scavenging ability of green-synthesized silver nanoparticles from *Plumeria alba*, supporting our findings. Inflammation or carcinoma is associated with elevated levels of NO in the body, which NO is produced by NO synthase enzymes. When superoxide radicals interact with NO during biochemical reactions, they form the highly reactive anionic peroxynitrite. The phenolic content and antioxidant activity of the biosynthesized FeNPs clearly show their potential for scavenging NO radicals and inhibiting inflammation or carcinoma.

Hemolytic activity

Hemolytic behavior is directly correlated with the role of drug circulation in the body and its healing power. The RBC membrane, composed of polyunsaturated fatty acids, is highly susceptible to oxidative stress, causing its disruption and the release of hemoglobin into the surrounding plasma. To rationalize their biomedical potential, the synthesized FeNPs were evaluated for hemolytic activity against RBCs. The green-synthesized FeNPs showed hemolysis ranging from 0.034% to 3.34%, whereas AE-Mk showed 0.076%–2.31% hemolysis at 10–100 µg/mL concentrations (Fig. 4(f)). These values indicate negligible toxicity within a specific limit, supporting the biomedical application of FeNPs. Conversely, Tritin X-100 caused 100% hemolysis, while PBS showed no hemolysis. Laloy et al. [64] studied the effects of silver nanoparticles on RBCs over time to determine toxicity level. Their findings showed nanoparticle safety within a specific limit, closely aligning with our results and reinforcing our findings.

Antimicrobial activity

One of the outstanding features of metal nanoparticles is their prominent antimicrobial properties, which vary across different microorganisms. The antibacterial effect of plant-mediated metal nanoparticles is attributed to the release of positively

charged salt ions from their partial oxidation in solution [5, 65].

The antibacterial activity of FeNPs showed the highest inhibition zone (19 mm) against *B. subtilis* at 500 µg/mL but a comparatively lower inhibition zone (16 mm) against *E. coli* at the same concentration. Under the same conditions, AE-Mk showed smaller inhibition zones against *B. subtilis* (15 mm) and *E. coli* (14 mm). Conversely, the standard chloramphenicol exhibited inhibition zones of 24 and 21 mm against *B. subtilis* and *E. coli*, respectively (Fig. 4(g)). Upon exposure to FeNPs, bacterial cell death might have occurred due to membrane damage. One hypothesis suggests that nanoparticles adhere to the bacterial cell surface, leading to cellular dysfunction. The positively charged bacterial cell surface facilitates the adsorption of positive metal ions. The synthesized metal nanoparticles also have a large surface area, which is quite suitable for more pathogenic cellular exposure. Metal nanoparticles exhibit different antibacterial effects on gram-positive and gram-negative bacteria. Some studies suggest that gram-positive bacteria are more susceptible than gram-negative due to differences in cell wall structure, whereas others report the opposite trend. Notably, biosynthesized nanoparticles often exhibit stronger antibacterial activity than their chemically synthesized counterparts. The exact role of phytochemicals in enhancing nanoparticle activity is still under consideration [66]. An antibacterial study involving iron oxide nanoparticles as drug carriers [67] demonstrated their significant role in drug delivery. In antifungal testing, FeNPs exhibited a notable inhibitory effect against *S. commune* (19 mm) and *A. niger* (13 mm) (Fig. 4(h)). Similarly, AE-Mk inhibited these strains to a lesser extent (15 and 14 mm against *S. commune* and *A. niger*, respectively). However, fluconazole showed 21 and 18 mm inhibitory zones against *S. commune* and *A. niger*, respectively, at 500 µg/mL. It should be noted that antimicrobial activity increased with higher nanoparticle concentrations. The biomedical efficacy of green-synthesized metal nanoparticles has been investigated regarding their antifungal potential, with many investigations reporting their strong antifungal activity [68]. Metal nanoparticles attach to the surface of hyphae, inactivating their function by releasing ions. Additionally, the antimicrobial activity of green-synthesized metal nanoparticles may rely on their interference with cellular replication, which is crucial

for cell survival. A general overview of the mode of action of these nanoparticles against biological stresses is presented in Fig. 5.

In vivo biocompatibility evaluation

Liver functionality

The liver plays a key role in maintaining overall health by performing numerous vital functions. Assessing the behavior of its enzymes in relation to foreign substances is crucial to understanding it. Therefore, bilirubin (total), AST, ALP, and SGPT levels were analyzed, with the results presented in Table 1. All values remained within the normal range. ALP and SGPT are key enzymes involved in detoxification and metabolism, while total bilirubin is a byproduct of heme catabolism. Together, they contribute to vital functions necessary for sustaining life and overall well-being. The findings indicate that FeNPs had no toxic effects on these biomarkers, providing valuable information.

The data were obtained through experiments involving three randomly selected animals per group (FeNPs-treated and control [PBS] groups). Results were analyzed using one-way ANOVA and presented

as mean $\pm$ S.E.

Histopathology

Biological systems naturally respond to foreign entities based on their nature and concentration. Therefore, assessing drug effects within biological systems is essential before advancing to formulation. FeNPs caused no toxicity at the cellular level, as shown in Fig. 6. The selected dose (30–100 $\mu\text{g/mL}$) created no visible lesions or damage to the liver, kidneys, heart, or lungs, as confirmed when compared with their respective controls under the same experimental conditions. These findings align with previous studies conducted by Mumtaz et al., which reported no toxic effects of AgNPs synthesized from *Murraya koenigii* on liver tissues, as well as biochemical markers, including bilirubin, ALP, and SGPT [3]. These results highlight the nontoxic nature of FeNPs in living subjects and support their potential for therapeutic applications.

In vitro cytotoxicity evaluation

The cytotoxicity effects of FeNPs on HeLa cell lines were evaluated using the MTT assay (Fig. 7). A significant reduction in cell viability was observed, decreasing from 90.12% to 58.23% (IC_{50} : 51.35) as

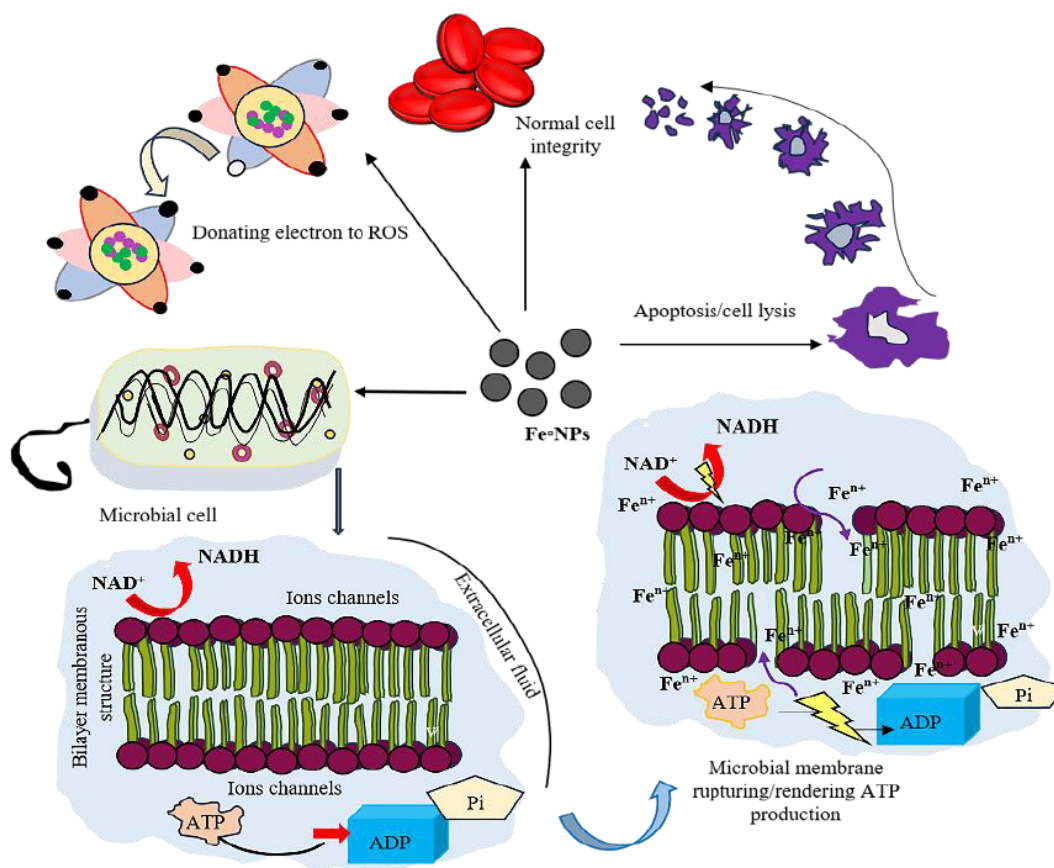
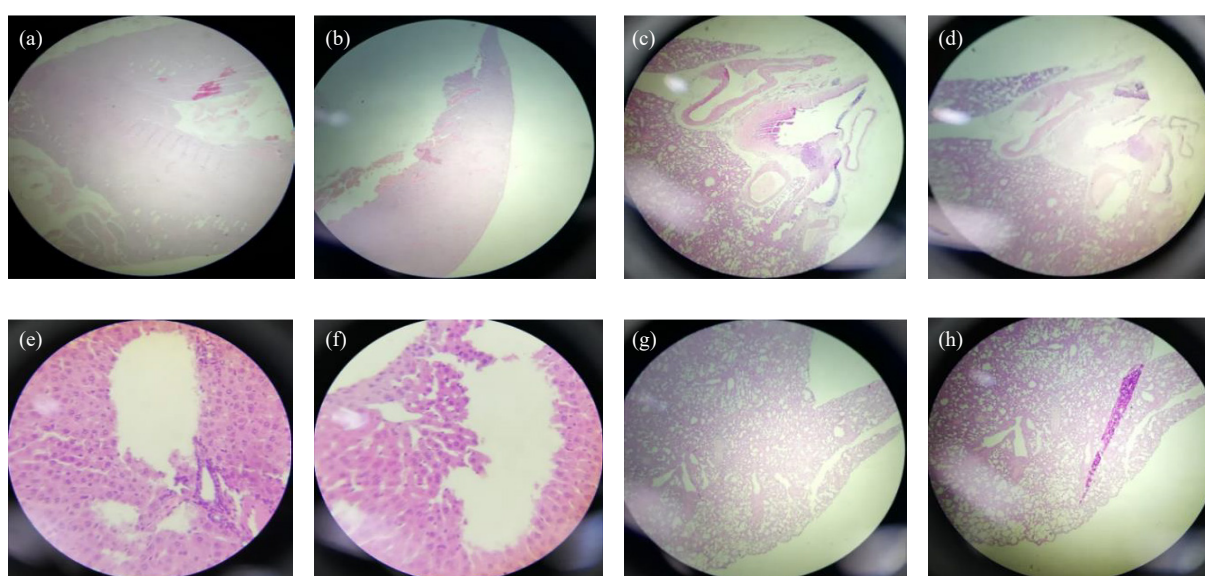


Fig. 5 Mechanism of FeNPs activity against ROS, drug-resistant microbial strains, and biocompatibility with healthy RBCs

Table 1 Biochemical integrity of the liver as a function of its enzymes and compounds on exposure to FeNPs over 28 days.

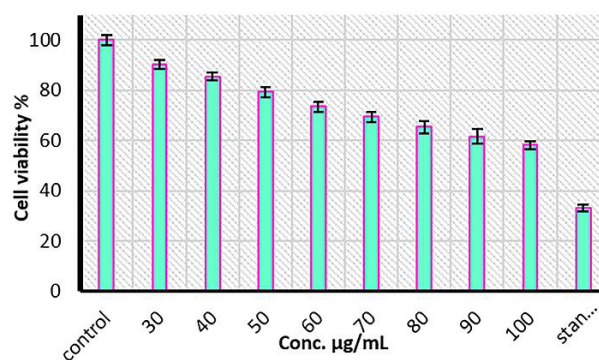
Liver biochemical parameters (enzymes)			
Biomarker	FeNPs-treated group (Mean $\pm$ S.E.)	Control group (Mean $\pm$ S.E.)	Normal range
ALP (IU/L)	59 $\pm$ 0.2	71 $\pm$ 0.3	80–306
AST (IU/L)	102 $\pm$ 0.4	88 $\pm$ 0.2	05–150
SGPT (IU/L)	22 $\pm$ 0.2	35 $\pm$ 0.2	09–43
Serum bilirubin (secreted compounds) (mg/dL)			
Direct	0.1 $\pm$ 0.01	0.09 $\pm$ 0.01	0.0–0.3
Indirect	0.5 $\pm$ 0.07	0.4 $\pm$ 0.02	0.0–0.8
Total	0.8 $\pm$ 0.02	0.5 $\pm$ 0.06	Up to 1.1

**Fig. 6** Biological compatibility of FeNPs in organs of Wistar rats over 28 days. FeNPs-treated group: (a) liver, (b) control group; (c) kidney, (d) control group; (e) heart, (f) control group; and (g) lung, (h) control group. Results were derived from three animals per group and compared with the control. Histopathological data were statistically analyzed and expressed as the mean of triplicate measurements.

the concentration increased from 30 to 100 μ L. The untreated control maintained 100% cell viability, whereas the standard drug (Doxorubicin) showed a 33.21% reduction in cell viability at 100 μ L. These findings provide remarkable evidence of the anticancer potential of FeNPs.

The anticancer activity on the different cell lines through the application of metal nanoparticles was evaluated by several researchers whose reports showed considerable cytotoxicity against these cells by significantly reducing cell survival. The probable cell diminishing involves the uptake of nanoparticles into cells through phagocytosis or pinocytosis. Once taken up by the cells, these nanoparticles accumulate and interact with cellular components, particularly DNA, causing damage and functional inactivation. In

tumor cells, cell death occurs due to apoptosis and necrosis, leading to the leaching of the cytoplasmic or

**Fig. 7** Cell viability analysis of FeNPs at varying concentrations. Data are presented as mean $\pm$ S.E. ($n = 5$), with significance levels compared to the standard ($p < 0.05$; $p < 0.001$).

nuclear contents from necrotic cells. Consequently, nuclear shrinkage occurs within condensed chromatin. This process highlights how phyto-reduced nanoparticles induce cell death [69].

Conclusions

The phytochemical-based synthesis of FeNPs was conducted using AE-Mk, a medicinal plant, through a single-step, self-sustaining process for evaluating *in vivo* and *in vitro* bioactivities. Synthesis was initially confirmed through UV–Vis spectroscopy (broad band at 620 nm) and further affirmed through FTIR spectroscopy, which identified functional groups such as alcohols, carboxylic acids, aliphatic primary amines, N–H stretches, alkanes, alkenes, and alkynes. Additional characterization techniques, including SEM, EDX, and XRD, revealed the polydisperse nature, elemental composition, and amorphous structure of the nanoparticles. Optimization studies ensured efficient synthesis and high FeNP yield. The antioxidant, antibacterial, and hemolytic activities were significant, representing strong biological functionality, including rapid radical scavenging potential, potent antimicrobial inhibitory effects, and nontoxic nature. Further evaluation of biocompatibility in Wistar rats showed no adverse effects in vital organs (heart, liver, kidney, and lungs), while *in vitro* cytotoxicity assays (MTT assay) against HeLa cell lines confirmed significant anticancer potential, with a 58.23% decrease in cell viability. Overall, these findings confirm the nano-biomedical potential of FeNPs and support their applicability in biomedicine.

Ethical Approvals

The animal procedure was approved and screened based on the ethical guidelines of the National Biosafety Committee and the Punjab Biosafety Rules, 2014, with approval from the Office of Research, Innovation & Commercialization, University of Agriculture, Faisalabad (Bioethics Certificate No. Chem 1321).

CRedit Author Statement

Sumaira Mumtaz: manuscript writing, drafting, methodology. **Raziya Nadeem:** supervision, cross-

checking, and data sequencing; **Ietaz Husain:** Data management, formatting, and analysis; **Sumaira Hanif:** data curation, editing; **Pir Muhammad:** data compilation; **Shazia Parveen:** data editing, reviewing; **Sadia Noor:** characterizing data; **Saeed Akhtar:** data formatting and correction.

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Conflict of Interests

There are no conflicts to declare.

Data Availability Statement

The data will be made available on request.

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